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COMMENTARY

The *In Vitro* Spheroid Melanoma Cell Culture Assay: Cues on Tumor Initiation?

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Cancer stem cells (CSCs) represent malignant subpopulations that initiate and maintain tumorigenic growth in hierarchically organized tumors via their considerable capacity for self-renewal and differentiation. CSCs have been identified in several human malignancies, including human malignant melanoma. Perego and colleagues' report in this issue indicates that CSCs capable of melanoma initiation in serial human-to-mouse xenotransplantation assays may be contained both among spheroid melanoma cell cultures (melanospheres) and among adherent melanoma cultures upon *in vitro* expansion. These results challenge the utility of the melanosphere assay as a surrogate tool for CSC identification in human melanomas and underline the importance of molecularly defined malignant melanoma initiating cells for CSC-focused diagnostic and therapeutic investigations.

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According to a consensus definition (Clarke, 2006), cancer stem cells (CSCs) are cells within hierarchically organized tumors that possess extensive self-renewal capacity and the ability to give rise to non-CSC progeny through differentiation. CSCs can be defined experimentally only by their ability to recapitulate the generation of a continuously growing tumor. Therefore, experimental identification and characterization of putative CSC subpopulations require the use of *in vivo* model systems (Clarke, 2006). The gold standard assay in this regard, although imperfect, is serial xenotransplantation of marker-defined clinical cancer subpopulations at limiting dilution into orthotopic sites of immunocompromised mice—typically nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Clarke, 2006) (Figure 1). Using this approach, CSCs have been identified in several human malignancies (reviewed in Frank, 2010), including human melanoma, in which malignant melanoma initiating cells (MMICs) are defined by expression of the chemoresistance mediator ABCB5 (Schatton, 2008). Hierarchical tumor organization has recently also been identified in syngeneic mouse models of melanoma, with only a fraction of murine melanoma cells possessing the fundamental CSC properties of extensive self-renewal, differentiation, and enhanced tumorigenic capacity (Held, 2010).

Perego *et al*. (2010, this issue) report that molecularly heterogeneous melanoma cells expanded *in vitro* as spheroid cultures (melanospheres) display more rapid growth *in vivo* in human-to-mouse xenotransplantation experiments compared with melanoma cells expanded as adherent cultures. At the same time, results of the study indicate that melanoma cells capable of tumor initiation in serial xenotransplantation experiments—i.e., MMICs—can be present both among spheroid melanoma cultures and among adherent melanoma cultures. These results challenge the potential utility of the melanosphere assay as a tool to enrich MMICs prospectively.

The sphere-formation assay was originally established as an *in vitro* culture system that enriches for mammalian stem cells of the central nervous system (reviewed in Jensen, 2006). Under these culture conditions, neural stem cells preferentially grow into so-called neurospheres that can differentiate into several neural lineage subtypes, whereas more differentiated cells die rapidly in response to mitogens present in the medium (Jensen, 2006). This *in vitro* culture system has since been adopted to assay for additional physiologic stem cell types, including mammary epithelial stem cells. Furthermore, some researchers have attempted to establish the sphere technique as a surrogate CSC assay, for example, for tumors of the brain and breast. However, the sphere assay suffers several disadvantages (Jensen, 2006): (i) it is very sensitive to the culturing method used (i.e., media composition and seeding technique), making it difficult to consolidate experimental findings from different laboratories; (ii) even at limiting-dilution plating, individual spheres often develop as a result of aggregation of cells rather than through clonal expansion; (iii) in some instances, more differentiated cells have been described as exhibiting sphere-forming capability; and (iv) because spheroid bodies themselves display marked cellular heterogeneity, experiments using spheres should be interpreted as studies of mixed cell populations, not purified stem cells. Taken together, these considerations have challenged the universal applicability of the sphere culture system as a stand-alone assay for the accurate enumeration of stem cell frequencies and for the study of stem cell biology in general (Jensen, 2006). To potentially make use of the sphere approach as a surrogate CSC assay, it has been posited that its reliability for the enrichment of tumor subpopulations of CSC phenotype and function must therefore be confirmed by stringent *in vivo* characterization of CSC activity (Clarke, 2006).

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Clinical Implications

- *In vitro* patterns (spheroid or adherent) of melanoma growth failed to predict molecular or functional phenotype.
- The results challenge the utility of the melanosphere assay as a surrogate tool for enriching cancer stem cells.
- The results underline the importance of molecularly defined malignant melanoma initiating cells for cancer stem cell–focused diagnostic and therapeutic studies.

These limitations of the sphere assay for CSC identification also apply to the study by Perego *et al*. (2010). For instance, whereas xenotransplantation of human melanoma cells derived from melanospheres to SCID mice resulted in significantly larger tumors compared with those observed in murine recipients of adherent melanoma cells, no significant differences in tumor-formation capacity were found between the two experimental groups (38/44 versus 36/47 xenograft tumors, respectively). In addition, both melanoma sphere–derived and adherent melanoma cell–derived xenografts could be passaged serially into secondary and tertiary recipients *in vivo*, demonstrating the presence of cancer cells with long-term self-renewal capability in both cell culture systems. Additionally, a lack of use of repurified melanoma cells prospectively identified by their ability to grow as spheroid bodies versus adherent populations in secondary and tertiary xenograft recipients in this study precludes the possibility of establishing any potential preferential association between CSC phenotype and sphere-forming ability or adherent phenotype. However, the results do show that both melanospheres and adherent melanoma cultures can contain tumor subpopulations with tumor-initiating, self-renewal, and proliferative capacity—i.e., CSCs. This conclusion might help to explain the observed lack in this study of significant correlations between candidate or known CSC markers with sphere-forming ability. Although the investigators reported increased expression of Nanog and Oct3/4 and decreased expression of the melanoma-differentiation antigen CD146 (MCAM) in spheroid versus adherent melanoma cultures, a specific relationship of these molecular markers to CSCs in human melanomas has not yet been established. Similarly, the capacity for transdifferentiation along mesenchymal lineages into cells with the morphology and antigenic properties of osteocytes, adipocytes, and chondrocytes (Perego *et al.*, 2010) observed among melanosphere-associated cells cannot be interpreted as being indicative of CSC phenotype, because tumor cell plasticity is not a defining feature of CSCs (Clarke, 2006). Moreover, melanosphere expression of the differentiation antigen MART-1 (Perego *et al.*, 2010) does not support the view that the melanosphere assay enriches for CSCs, because MART-1 is expressed only at relatively low levels by MMICs (Schatton, 2010).

The finding by Perego *et al*. that CSCs in human melanomas can be contained both among spheroid melanoma cultures and among cultured adherent melanoma cells is novel and extends previous investigations by other laboratories that have sought to address a potential correlation of *in vitro* melanoma growth patterns (i.e., spheroid versus adherent growth) with the CSC phenotype of human melanomas (Fang, 2005; Keshet, 2008; Monzani, 2007). It should be noted that these other studies did not utilize serial *in vivo* xenotransplantation assays for the investigation of candidate CSC subpopulations and therefore fell short of yielding definitive insights in this regard. Monzani *et al*. (2007) found that tumorigenic CD133$^+$ melanoma subsets were contained exclusively among adherent melanoma populations, whereas melanoma sphere–associated cells did not express CD133 (Monzani, 2007), in contrast to the current observation by Perego *et al*. (2010). Fang *et al*. (2005) reported increased expression of the B-cell marker CD20 on melanoma spheres compared with adherent melanoma cells, but the significance of this result remains uncertain because CD20 expression was not detected in either sphere-forming or adherent melanoma cultures (Perego *et al.*, 2010) or clinical melanoma specimens (Quintana *et al.*, 2008). Keshet *et al*. (2008) described increased expression of the multidrug-resistance gene product MDR1 by melanomas under melanosphere-forming culture conditions compared with

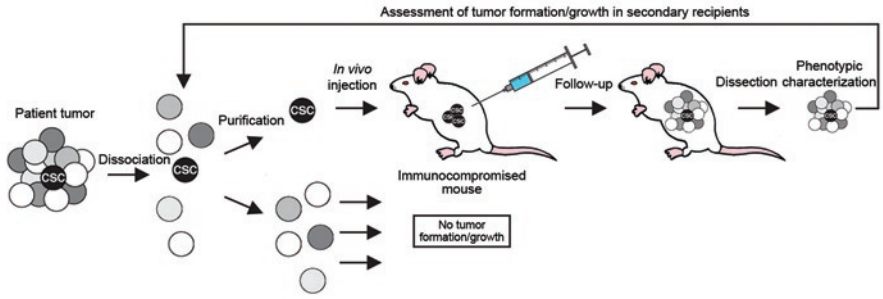

Figure 1. The gold standard assay for the study of cancer stem cells (CSCs). A CSC is a cell within a tumor that possesses the capacity to undergo both self-renewing cell divisions and cell divisions that result in more differentiated cancer cell progeny. CSCs can be defined experimentally only by their ability to recapitulate the generation of a continuously growing tumor. The gold standard assay that fulfills this criterion is serial xenotransplantation at limiting dilution of marker-defined clinical cancer subpopulations into an orthotopic site of immunocompromised (typically nonobese diabetic/severe combined immunodeficient (NOD/SCID)) mice. In this assay, long-term self-renewal of CSCs is confirmed by serial xenotransplantation of prospectively reisolated CSC populations into secondary recipients. Differentiation of CSCs is typically demonstrated by their ability to generate tumor xenografts that reflect the cellular heterogeneity of the original patient tumor. Modifications to this assay system, including the exogenous addition of growth and extracellular matrix factors, may complicate biological readouts because they could enable non-CSCs to contribute to tumor growth.

adherent culture conditions. In contrast, Perego *et al*. (2010) found that MDR1 mRNA was not differentially expressed in melanospheres compared with adherent melanoma cultures.

In aggregate, the currently available evidence does not support a significant or preferential correlation of a particular *in vitro* melanoma growth pattern (i.e., spheroid or adherent growth) with known melanoma CSC molecular phenotypes or functions. These results challenge the utility of the melanosphere assay as a surrogate tool for enriching CSCs in human malignant melanoma and underline the importance of molecularly defined MMICs for CSC-focused diagnostic and therapeutic investigations.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Vexed by Red-Headed Conundrums

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Reduced function alleles of the melanocortin 1 receptor (MC1R) lead to red hair, freckling, and sun sensitivity. They are also associated with an increased risk of skin cancer. But what pathways link gene variants with the cancer phenotype? In this issue, Robinson *et al.* describe their use of mouse transgenics to demonstrate that the MC1R signaling pathway influences cancer risk via mechanisms in addition to pigmentation.

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"…and then off to violin-land, where all is sweetness…and there are no red-headed clients to vex us with their conundrums."
—Sherlock Holmes,
in "The Red-Headed League"

In Arthur Conan Doyle's Sherlock Holmes story "The Red-Headed League," the central character, Jabez Wilson, has fiery red hair. Ensnared by bank robbers, he is persuaded to leave his shop so that he can take up generously paid work elsewhere. Curiously, this well-paid work is open only to those with red hair, and the work itself appears to serve no useful function. The reader is left wondering what aspect of red hair is critical to the logic of the story, but the explanation is mundane. We learn that Wilson's shop is situated next to a bank, and the villains want him out of the way so that they can tunnel into the bank next door. The story could therefore have focused on any of Wilson's characteristics, and red hair was used merely as a marker.

What of red hair and skin cancer? We know that most forms of skin cancer are more common in individuals with red hair and pale skin (Rees, 2004). Does causality run from altered pigmentation to skin cancer, or, as in the story, is red hair merely a visible marker for some other characteristic? The paper by Healy's group (Robinson *et al.*, 2010, this issue) provides us with yet more reasons to admit that the link between red hair and skin cancer still vexes.

The evolution of human pigmentation

Human pigmentation attracts great interest, and not only because of its link with skin disease. Variation in human skin and hair color is one of the most striking polymorphic traits of mankind (Rees, 2004). Covariation of skin color, ancestry, and geography is obvious to most observers and has played a key role in human history—for good and bad. If we were to précis human evolution over the past 500,000 years, it would perhaps read as follows.

Following climate change, our ancestors were forced to move from the forest to the savannah. The need to hunt meant that endurance exercise became essential—we may not have been able to outsprint other mammals, but humans can still outrun a horse over marathon distances in a warm climate. We were therefore able to track and hunt prey over large distances. We owe much of this ability to the way our skin is designed. Humans are covered by 3 to 5 million eccrine glands, and we are astonishingly capable of losing body heat generated by physical activity. However, sweating was more efficient if evaporation could take place unimpeded by hair; thus humans became—to use the cliché—naked. This in turn led

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